maximum medical treatment, including i.v. inotropes. The aortic valve was directly closed with a running 5-0 polypropylene suture under cardiac arrest.

Two patients with Toyobo biventricular support (patients 4 and 5) received conversion to 2 implantable devices: the DuraHeart as left ventricular support and a Jarvik 2000 axial-flow pump as right ventricular support.[15] The Toyobo inflow cuff in the right ventricular free wall had to be replaced with a Jarvik 2000 inflow cuff because of the size difference. Care was taken to position the Jarvik 2000 pump towards the tricuspid valve to avoid flow obstruction by sucking on the right ventricular wall or interventricular septum. The anterior leaflet of the tricuspid valve was excised or sutured to the ventricular free wall. In patient 4, direct closure of the pulmonary valve was also carried out for severe pulmonary insufficiency.

The operative times and postoperative course are summarized in **Table 2**. The mean operation time of the 5 patients who underwent isolated LVAD conversion was 318±54 min (range, 251–387 min). The mean CPB time of those patients was 65±27 min (range, 39–103 min).

There was no hospital mortality. Chest re-exploration was required for bleeding in 2 patients who underwent conversion to implantable BiVADs. All patients, except patient 5, who required temporal tracheostomy, were extubated on postoperative day 1 or 2. All patients were discharged following a mean postoperative hospital stay of 77 days.

The mean duration of DuraHeart support was 388 days (range, 30–631 days). No neurological complication or significant hemolysis was noted in all patients. One patient (patient 6) successfully received a heart transplant 176 days after the conversion. Six patients are now at home awaiting heart transplantation.

Two patients (patients 2 and 3) developed pump pocket infection. They had a limited local infection or minor erosion around the skin exit site of the Toyobo (**Figure 2A**), and operative findings indicated no major abscess around the inflow/outflow cannulae. It was necessary for them to undergo an omental flap procedure, however, 2 and 6 months after conversion, respectively. At the time of writing they had no recurrence of infection for 11 months and 19 months, respectively.

One patient (patient 5) died 10 months after the operation. This patient underwent conversion to the implantable BiVAD (DuraHeart and Jarvik 2000) after 224 days of Toyobo BiVAD support. This patient had methicillin-resistant *Staphylococcus aureus* infection of the Toyobo cannula exit site prior to conversion (**Figure 2B**). Computed tomography (CT) showed only local infection around the cannula exit site but, during the operation, the infection was found to have reached the Toyobo RVAD inflow cuff and aggressive debridement and an omental flap procedure were added. The postoperative course was uneventful and she was discharged with no sign of infection. This patient was re-hospitalized, however, 10 months after surgery because of the acute onset of spiking fever and eventually died from sepsis. Autopsy showed extensive mediastinitis and pump pocket infection.

One patient (patient 6) experienced a device malfunction and required a device exchange after 259 days of DuraHeart LVAD support. Failure of magnetic levitation occurred through a fracture on a position sensor wire in the percutaneous cable. Device exchange was successfully performed using a subcostal approach without a repeat sternotomy.[16]

Discussion

Although heart transplantation remains the gold-standard therapy for end-stage heart failure, the severe donor organ shortage in Japan forces patients on LVAD support to wait >2 years for transplantation. The Toyobo paracorporeal LVAD, which has been the only device covered by Japanese national insurance for a long time, has many problems in long-term use. We have previously reported that the actuarial survival rate has been improved recently, but actuarial survival rate of patients on Toyobo LVAD support in 2003–2007 was 66.3% at 6 months and 45.9% at 1 year.[11] We have also reported that Toyobo LVAD was the independent risk factor for device-related infection, and freedom from LVAD-related infection was only approximately 30% at 6 months and 20% at 1 year. Freedom from cerebral stroke events was approximately 50% at 6 months and 35% at 1 year.[12]

To resolve this unacceptable situation in Japan, clinical trials of 4 implantable continuous-flow LVADs (DuraHeart, EVAHEART, Jarvik 2000, HeartMate II) have been carried out in Japan, and the results of these trials were favorable. A recently conducted multi-center, randomized controlled trial in the USA demonstrated the superiority of the implantable continuous-flow device compared to the first-generation pulsatile device.[7] DuraHeart is the world's first approved, magnetically levitated centrifugal left ventricular assist system, which eliminates all mechanical contacts between the impeller and the drive mechanism, thereby providing superior durability, with reduced likelihood of thrombus and hemolysis. In an early clinical study managed in Europe, the adverse event rates per patient-years for major adverse events during DuraHeart support were acceptable in comparison with those of the second-generation axial-flow LVADs.[6,12,13] Morshuis et al reported that the driveline or pocket infection rate of DuraHeart LAVD was 0.27 per patient-year, which was reduced by 90% compared with that of HeartMate VE, the pulsatile device, and was comparable to that of HeartMate II, a small axial-flow device. The rate of neurological events in DuraHeart was 0.21 per patient-year, which was 50% less than that of HeartMate VE and comparable to that of HeartMateII.[1,4,17]

Considering these recent outcomes, it is expected that conversion to implantable LVAD from Toyobo LVAD will provide better survival if the operation is performed safely. In addition, a patient with an implantable LVAD can be discharged home. This holds great advantages for QOL and health economics.

In the conversion procedure, the apical cuff of the Toyobo VAD can be used with the DuraHeart inflow conduit, so it is not necessary to replace the Toyobo apical cuff with that of the DuraHeart, which allows operation time to be shortened and the risk of bleeding reduced. This is especially beneficial in pure LVAD conversion. In 5 cases of isolated LVAD conversion (patients 1, 2, 6, 7, 8), the mean CPB time for those patients was relatively short, and all conversion operations were performed safely with no patients requiring re-exploration for bleeding. For pure LVAD conversion, we consider the operative risk to be relatively low. In the BiVAD conversion, however, operative risk is much higher because some additional procedures are required.[15] The present 2 patients required re-exploration for bleeding and prolonged ventilation, but they could be discharged home finally. Considering the poor clinical results of Toyobo BiVAD support, although certain risks remain, conversion to implantable VADs is beneficial for these patients.

Although certain risks of the conversion operation may still remain, we consider the risk of continuing Toyobo device for >1 year to be higher than the operative risk. This strategy, however, also carries a certain risk. In the present series, 3 of

the 8 patients developed device pocket infection. We usually sterilize cannula site every day and in the case of minor erosion or focal infection, repeated culture are examined. If there is an active infection with pus or abscess around the exit site, daily lavage with saline is added. But, as previously mentioned, we reported that Toyobo LVAD was one of the independent risk factors for device-related infection.[11] We have also reported that device-related infections at various locations were observed in 68.9% of all Toyobo patients. Inflow/outflow cannula exit site infection was observed in 31.1% of patients. Although the infectious events occurred most frequently in the first 6 months after LVAS implantation, events continued to occur thereafter. The infection-free rate improved by year of implantation, but the infection-free rate was approximately 70% at 6 months and only 50% at 1 year in 2003–2007.[11] Therefore, careful attention must be paid to Toyobo exit site infection prior to conversion. One patient (patient 3) had minor tissue erosion around the exit site of the Toyobo cannula and the other 2 patients (patients 2 and 5) had local infection of the Toyobo cannula exit site prior to conversion. Preoperative CT and laboratory data showed no sign of systemic infection or mediastinitis in all patients. During the conversion operation, the skin exit site was carefully removed and aggressive debridement performed if any local infection was noted during the operation. Nevertheless, all 3 patients developed pocket infection. In the debridement and omental flap procedures, a large amount of abscess in the pump pocket was found in all 3 patients. Two patients recovered without any recurrence but 1 patient with biventricular support died from uncontrollable sepsis. Even a superficial infection of the cannula exit site is considered to be a significant risk for developing refractory pocket infection after conversion, which leads to fatal complications. From these experiences, we now consider this procedure to be a contraindication if a patient has any signs of infection at the Toyobo LVAD cannula exit site.

Conclusion

We have reported our initial experience with conversions from the Toyobo LVAD to the DuraHeart. The procedure was performed safely, and clinical outcomes were satisfactory. The replacement of Toyobo LVADs with DuraHeart LVADs will be highly advantageous in patients with Toyobo LVADs without any device infection.

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The use of cell-sheet technique eliminates arrhythmogenicity of skeletal myoblast-based therapy to the heart with enhanced therapeutic effects ☆

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ABSTRACT

Background: Clinical application of skeletal myoblast transplantation has been curtailed due to arrhythmogenicity and inconsistent therapeutic benefits observed in previous studies. However, these issues may be solved by the use of a new cell-delivery mode. It is now possible to generate "cell-sheets" using temperature-responsive dishes without artificial scaffolds. This study aimed to validate the safety and efficacy of epicardial placement of myoblast-sheets (myoblast-sheet therapy) in treating heart failure.
Methods and results: After coronary artery ligation in rats, the same numbers of syngeneic myoblasts were transplanted by intramyocardial injection or cell-sheet placement. Continuous radio-telemetry monitoring detected increased ventricular arrhythmias, including ventricular tachycardia, after intramyocardial injection compared to the sham-control, while these were abolished in myoblast-sheet therapy. This effect was conjunct with avoidance of islet-like cell-cluster formation that disrupts electrical conduction, and with prevention of increased arrhythmogenic substrates due to exaggerated inflammation. Persistent ectopic donor cells were found in the lung only after intramyocardial injection, strengthening the improved safety of myoblast-sheet therapy. In addition, myoblast-sheet therapy enhanced cardiac function, corresponding to a 9.2-fold increase in donor cell survival, compared to intramyocardial injection. Both methods achieved reduced infarct size, decreased fibrosis, attenuated cardiomyocyte hypertrophy, and increased neovascular formation, in association with myocardial upregulation of a group of relevant molecules. The pattern of these beneficial changes was similar between two methods, but the degree was more substantial after myoblast-sheet therapy.
Conclusion: The cell-sheet technique enhanced safety and therapeutic efficacy of myoblast-based therapy, compared to the current method, thereby paving the way for clinical application.

1. Introduction

Despite pre-clinical evidence showing that transplantation of skeletal myoblasts (SMBs) greatly improves the function of damaged hearts mainly via the paracrine effect [1], the use of this cell type in clinical cell therapy has been largely curtailed. This was mainly due to two adverse findings in previous clinical studies: occurrence of fatal ventricular arrhythmias and insufficient or inconsistent therapeutic effects [1,2]. We speculate that these issues were associated with the use of a suboptimal cell-delivery method, and that application of a more suitable method may solve both of these concerns.

The commonly used cell-delivery method in previous studies is direct intramyocardial injection of trypsin-treated SMB suspensions [1,2]. This method is, however, known to produce islet-like localized cell-clusters, which could cause disturbance of the electrical conduction, leading to re-entrance arrhythmias [3–5]. In addition, this method is associated with considerable donor cell loss by initial leakage and by cell death/damage due to injection-mediated mechanical injury and subsequent myocardial inflammation. Additional donor cell damage is caused by the enzymatic digestion (i.e. trypsinization) used for cell collection from culture dishes [5–7]. Trypsinization disrupts cell surface proteins and destroys cell–cell connections, thus deteriorating donor cell viability and functionalities. These adverse effects would collectively result in poor donor cell engraftment, which will consequently limit the benefit from this approach [1].

Recent development of the unique culture dish coated with a temperature-responsive polymer (poly-N-isopropylacrylamide) has enabled fabrication of "cell-sheets" simply by reduction of the temperature without any harmful chemical treatment and without using artificial

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scaffolds [7]. At 37 °C this polymer is hydrophobic, and cells can adhere to the dishes and grow. However, when the temperature is dropped to 25 °C or below, the polymer rapidly becomes hydrophilic, hydrated and swollen, losing its cell-adhesiveness. As a result, the cells detach from the dish as a free cell-sheet. In contrast to trypsinization, cell surface proteins, cell–cell junctions and underpinning extracellular matrix (ECM) are well preserved in this method. Following epicardial placement, cell-sheets are expected to quickly adhere to the heart due to the preserved ECM, minimizing donor cell leakage. Taken together, the epicardial placement of SMB-sheets (SMB-sheet therapy) is likely to achieve greater retention, survival, and engraftment of donor SMBs in the heart while maintaining important donor cell functionalities including the secretion of paracrine mediators, resulting in augmentation of therapeutic benefits, compared to intramyocardial injection. In addition, this innovative method will not produce intramyocardial tissue disruption that disturbs the electrical conductance, and therefore might prevent occurrence of ventricular arrhythmias. In fact, therapeutic effects of SMB-sheet therapy have been reported in various models [8–10]. However, more detailed pre-clinical investigations, particularly on arrhythmia occurrence and other factors concerning the safety and effects, are needed for this approach to be widely established in the clinical arena.

2. Materials and methods

All animal studies were performed with the approval of the institutional ethics committee and the Home Office, UK. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [11]. All procedures were carried out in a blind manner whenever possible.

2.1. Generation of SMB-suspensions and SMB-sheets

SMBs were collected from male Lewis rats (150–175 g, Charles River) by the single fiber method as previously described [5,12]. To generate an SMB-sheet, 4×10^6 SMBs (passage 4–5) were seeded on a 35-mm temperature-responsive culture dish (UpCell, CellSeed, Inc.). Following 12–15 h incubation at 37 °C, the temperature was lowered to 22 °C, enabling the SMB-sheet to detach from the dish [8]. For injection, 4×10^6 SMBs were collected using trypsinization and suspended in 200 μl PBS [5]. The size of generated SMB-sheets was approximately 15 mm in diameter. For graft tracking studies, SMBs were labeled with CM-DiI (Molecular Probes) according to the manufacturer's protocol.

2.2. Induction of myocardial infarction (MI) and SMB transplantation

Female Lewis rats (180–200 g, Charles River) underwent left coronary artery ligation as previously described [3,5]. The animals were randomly assigned to receive either SMB-sheet therapy (Sheet group), intramyocardial SMB injection (IM group), or sham-treatment (Cont group). For the Sheet group, an SMB-sheet was epicardially placed to cover the left ventricular (LV) free wall including both infarct and border areas. For the IM group, SMB suspension was injected into 2 sites (100 μl each) of the LV free wall, aiming to target a similar area to SMB-sheet therapy [3,5].

2.3. Measurement of arrhythmia occurrence

Incidence of spontaneous arrhythmias, including premature ventricular contraction (PVC), ventricular tachycardia (VT) and ventricular fibrillation (VF), was continuously monitored by a radio-telemetry system (Data Sciences International) as described previously [3,5]. For accurate evaluation of the arrhythmia severity, the modified Curtis and Walker's scoring system [13] was applied, where frequencies of PVC, VT and VF were systematically taken into account.

2.4. Histological analysis

At chosen time points, the hearts were excised, fixed with 4% paraformaldehyde, and frozen. Cryosections were cut and incubated with polyclonal anti-cardiac troponin-T antibody (1:200 dilution, HyTest), biotin conjugated *Griffonia simplicifolia* lectin I-isolectin B_4 (1:100, Vector), monoclonal anti-CD45 antibody (1:50, BD), monoclonal anti-CD11b antibody (1:50, Chemicon), monoclonal anti-granulocyte antigen (1:20, AbD Serotec), monoclonal OX62 (1:25, AbD Serotec), polyclonal CD3 (1:100, Abcam), or monoclonal connexin43 (Cx43; 1:250, Millipore), followed by visualization using appropriate fluorophore-conjugated secondary antibodies with or without nuclear counter-staining using 4′,6-diamidino-2-phenylindole (DAPI). Ten different fields from each of the border and remote areas per heart were randomly selected and assessed. Another set of sections were stained with 0.1% picrosirius red for assessing infarct size and for detecting collagen deposition [3,5]. To evaluate the cardiomyocyte size, the cross-sectional area of appropriately detected cardiomyocytes [14] was measured of 50 cardiomyocytes in each border and remote area per heart.

2.5. Evaluation of cardiac performance

Cardiac function and dimensions, and hemodynamic parameters were measured by using echocardiography (Vevo-770, VisualSonics) and cardiac catheterization (SPR-320 and PVAN3.2, Millar Instruments) by a blinded operator as previously described [3,5,14].

2.6. Analysis for donor cell survival in the heart and other organs

DNA was extracted from the heart, lung, liver, kidney, and spleen post treatment. The presence of male cells in each female organ was quantitatively assessed to define donor cell presence using real-time PCR (Prism 7900HT, Applied Biosystems) for the Y-chromosome-specific *sry* gene as previously described [3,5]. Non-heart organs that were positively detected for *sry* expression were defined to be ectopic donor cell survival.

2.7. ELISA for myocardial IL-1β levels

Proteins were extracted from the homogenates from frozen whole LV samples collected at day 3 post-treatment with lysis buffer (0.15 M NaCl, 1 mM EDTA, 20 mM Tris pH 7.4, 1 mM DTT and protease inhibitor cocktail (Sigma)). After measuring the protein concentration (BioRad DC protein assay), levels of IL-1β were measured using an ELISA kit (eBioscience) according to the company's instruction.

2.8. Analysis for myocardial gene expression

Total RNA was extracted from frozen whole LV samples and assessed for myocardial gene expression of genes presumably relevant to the SMB-mediated paracrine effect by quantitative RT-PCR (Prism 7900HT) as previously described [15]. TaqMan primers and probes were purchased from Applied Biosystems. Expression was normalized using *Ubiquitin C*.

2.9. Statistical analysis

All values are expressed as mean ± SEM. Statistical comparison of the data was performed using the Student's unpaired *t*-test for the donor cell survival in the heart and using χ^2-test for the ectopic donor cells. Other data were analyzed with one-way ANOVA followed by Fisher's post-hoc analysis to compare groups. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Eliminated arrhythmia occurrence by SMB-sheet therapy

We established a rat model to investigate whether the use of the cell-sheet technique might reduce arrhythmogenicity associated with SMB transplantation. Continuous electrocardiogram monitoring using radio-telemetry revealed frequent episodes of premature ventricular contractions (PVCs) in the IM group at both day 1 and 28 (Fig. 1A, C) consistent with previous clinical and experimental reports [1,5], validating the suitability of this model. There was a smaller number of PVC occurrence observed in the Cont group, which is also commonly seen post-MI [5,12], and more importantly the frequency of PVCs in the Sheet groups was just comparable to this base-line data. Furthermore, the IM group showed more frequent ventricular tachycardia (VT) occurrence than other groups; more than one-third of the animals in the IM group developed VT (Fig. 1B, D). Furthermore, one animal of the IM group developed transient ventricular fibrillation (VF) at day 28. In contrast, there was no animal that developed VT or VF in the Sheet group throughout the period studied. Consequently, the IM group, but not Sheet group, showed a higher arrhythmia score than the Cont group (Fig. 1E). These data are the first quantitative evidence that SMB transplantation-induced arrhythmogenicity can be prevented by the use of the cell-sheet technique.

3.2. Donor cell behaviors after SMB-sheet therapy

To gain an insight of the mechanism by which SMB-sheet therapy attenuated arrhythmogenicity, we first assessed the quantitative donor cell survival (presence). As a result, the donor cell presence in the heart in the Sheet group was 3.5-fold and 9.2-fold higher than that in the IM

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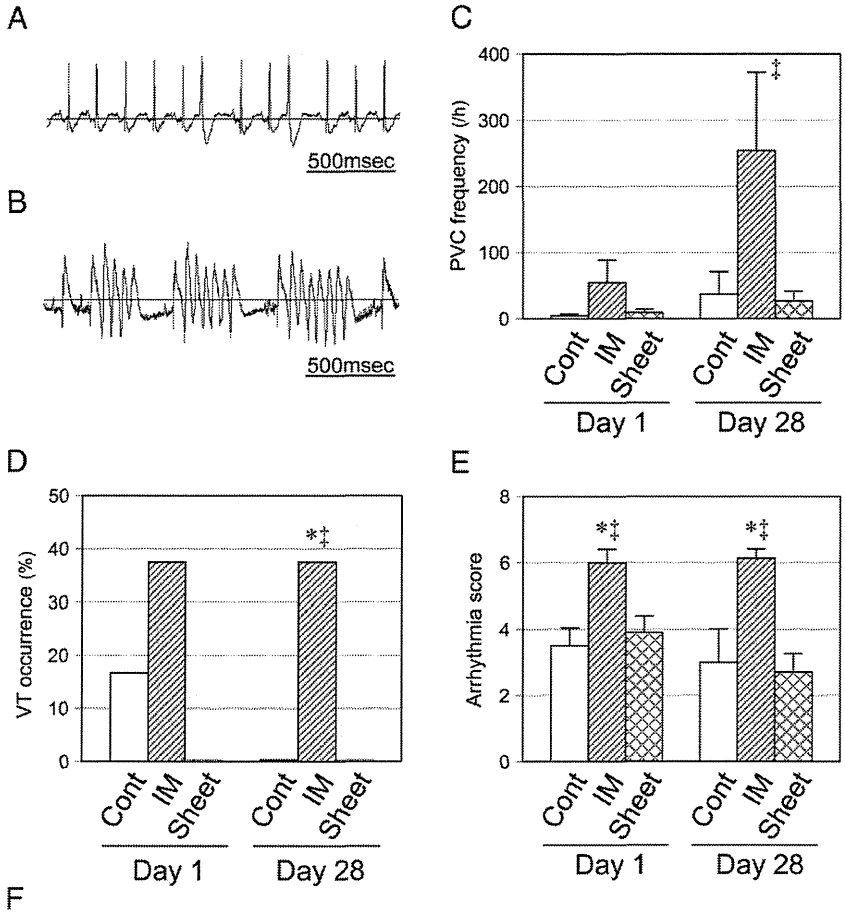

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	RR (msec)	P (msec)	PR (msec)	QRS (msec)	QTc (msec)
Cont	148.3±9.6	18.3±1.3	42.1±0.8	18.1±0.4	5.8±0.4
IM	159.2±11.0	20.7±2.0	43.5±1.3	20.1±0.7*‡	5.9±0.1
Sheet	155.8±7.5	19.6±0.7	43.1±1.3	17.1±0.4	5.4±0.1

Fig. 1. Eliminated arrhythmogenicity by SMB-sheet therapy. Arrhythmia occurrence was assessed by continuous electrocardiogram monitoring using radio-telemetry. Representative patterns of PVC (A) and VT (B) in the IM group at day 28 are shown. The IM group, but not the Sheet group, increased the occurrence of PVC (C) and VT (D; % animals) and consequently elevated the arrhythmia score (E; accumulated hourly scores for 6 h), in association with elongated QRS duration, at day 28 (F), compared to the Cont group. *$p<0.05$ vs. Cont group, ‡$p<0.05$ vs. Sheet group, $n=6$–8 in the Cont, IM, and Sheet groups, respectively.

group at day 3 and 28, respectively (Fig. 2A). This meant that SMB-sheet therapy achieved much greater SMB existence in the heart, but contradictorily reduced arrhythmia occurrence, suggesting that changes in the donor size was not a reason for the eliminated arrhythmogenicity after SMB-sheet therapy. We then looked into the differences in distribution and associated behaviors of SMBs between two methods by histological studies with tracking CM-DiI-labeled SMBs. As expected from previous studies [5], the IM group formed islet-like, localized cell-clusters composed of donor SMBs and host $CD45^+$ inflammatory cells at day 3 (Fig. 2B, F), which persisted up to day 28 with a reduced size (Fig. 2C, G). In addition, immunolabelling for Cx43 demonstrated that grafted SMBs expressed Cx43 protein (though to a limited extent) within the cell-clusters at day 3 in the IM group (Fig. 2J), but there was no Cx43-containing gap junction formation between SMBs and cardiomyocytes on day 3 or 28 (Fig. 2J, K). In contrast, the donor cell distribution was markedly different after SMB-sheet therapy; the majority of donor cells remained on the epicardial surface at both day 3 and 28 in the Sheet group, thus obviating the induction of myocardial heterogeneity or disruption (Fig. 2D, E, H, I). Also, there was no gap junction formation between SMB-sheets and cardiomyocytes after SMB-sheet therapy (Fig. 2L, M). Collectively, intramyocardial SMB injection caused the donor cell clusters with intense inflammation within the myocardium, which was electrically isolated from host cardiomyocytes, causing physical disturbance to electrical impulse propagation, and resulting in conduction delay, block, and eventually re-entrance arrhythmias. In contrast, SMBs were localized on the epicardial surface (outside myocardium) after cell-sheet placement and did not affect the electrical conductance of the host heart. Corresponding to these findings, the QRS duration was elongated at day 28 in the IM group compared to other groups (Fig. 1F).

3.3. Prevention of exacerbation of myocardial inflammation by SMB-sheet therapy

Another possible mechanism by which SMB transplantation induces ventricular arrhythmias may be an increase in the arrhythmogenic substrates, including inflammatory response. Post-MI failing cardiomyocytes

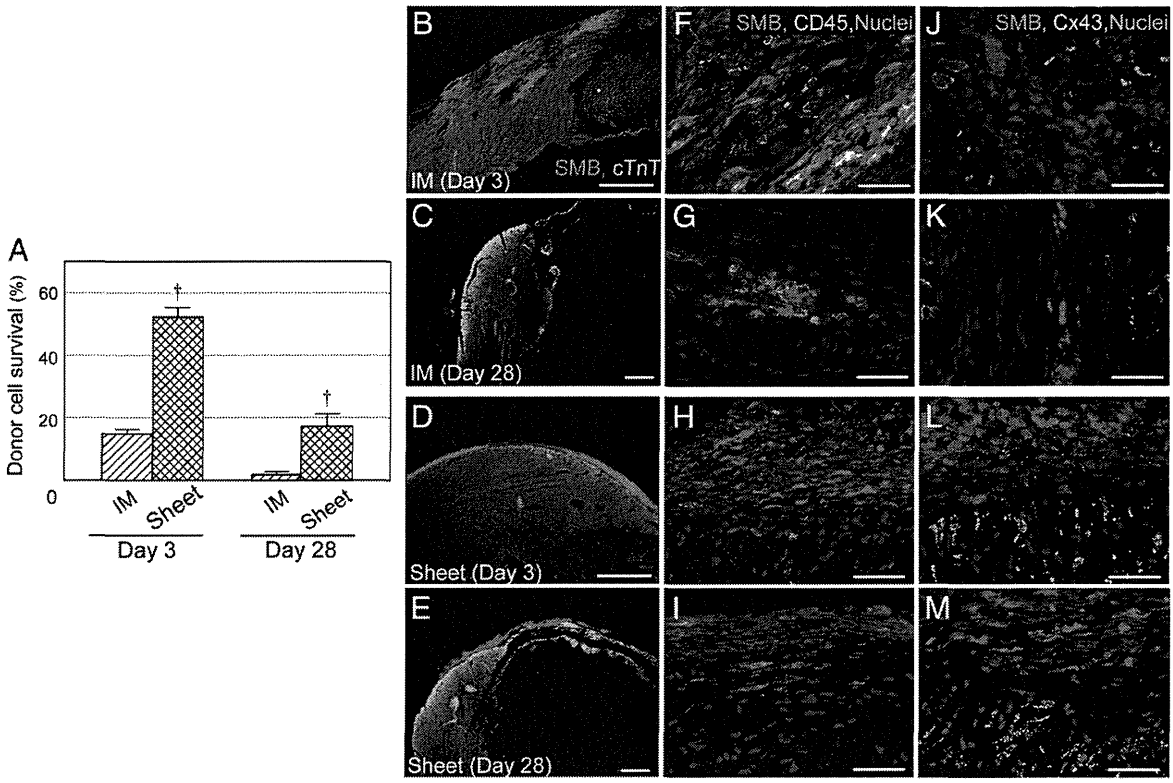

Fig. 2. Donor cell behaviors after SMB-sheet therapy. Quantitative assessments showed that donor cell survival (% of donor cell number existing in the heart) was higher in the Sheet group than in the IM group at both day 3 and 28 (A). †$p<0.01$ vs. IM group, $n=4$–6 in each point. Immunofluorescence showed that clusters of SMBs and CD45$^+$ inflammatory cells were formed in the IM group at day 3 (B, F) and 28 (C, G), while these intramyocardial heterogeneities were absent in the Sheet group, in which most of donor cells retained on the epicardial surface (D, H for day 3; E, I for day 28). Immunolabelling for Cx43 showed that there was no obvious gap junction formation between SMBs and host cardiomyocytes in both the IM (J, K) or Sheet group (L, M) at either day 3 or 28. Orange signals for SMBs (CM-DiI); blue for nuclei (DAPI); green for cardiac Troponin-T (cTnT) in (B–E), for CD45 in (F–I) or for Cx43 (J–M). Scale bar = 1 mm in (B–E) and 30 μm in (F–M). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

suffer adverse alterations of cellular properties including inappropriate gap junction expression/distribution and electrical instability, which will increase vulnerability to arrhythmogenic stimuli [16]. Our immunolabeling analyses demonstrated that there was increased accumulation of CD45$^+$ inflammatory cells globally into the host myocardium post MI, both at border and remote areas, in the Cont group (Fig. 3A). More importantly, this inflammatory response was further exacerbated in the IM group widely and persistently, while in contrast this exacerbation was prevented in the Sheet group. The IM group increased the accumulation of granulocytes (Fig. 3B), CD11b$^+$ monocytes/macrophages (Fig. 3C), and OX62$^+$ dendritic cells (Fig. 3D) into the border areas at both day 3 and 28, compared to other groups. Correspondingly, the myocardial level of a major pro-inflammatory cytokine, IL-1β, was significantly increased in the IM group, compared to the Cont group, whereas this increase was entirely absent after SMB-sheet therapy (Fig. 3E). IL-1β is known to increase arrhythmia susceptibility by increasing the Ca_2^+ leak from the sarcoplasmic reticulum [17]. These data collectively showed that intramyocardial SMB injection exacerbated inflammation and increased susceptibility of arrhythmias, while this adverse effect was obviated by SMB-sheet therapy.

3.4. Attenuated donor cell leakage into the lung by SMB-sheet therapy

During this study, we unexpectedly found another safety concern associated with intramyocardial SMB injection. PCR for the *sry* gene in various organ samples detected a substantial presence of male donor cells in the lungs of all host female animals at day 28 in the IM group (Fig. 4A). Consistent with this, donor cell trafficking studies using CM-DiI-labeled SMBs uncovered that there were a considerable number of donor cells globally distributing in the lung, forming discrete loci (Fig. 4C, D). It has been reported that many donor cells leak into other organs, commonly the lung and kidney, at early phases of intramyocardial injection of bone marrow-derived cells [18]. This aspect in the case of SMBs remains largely unexplored, and our study clarified that after intramyocardial SMB injection a considerable number of donor cells leaked and survived in the lung for at least 28 days. In contrast, in the Sheet group, no donor cells were detected in any organ studied, by either PCR or histological study (Fig. 4A, B), shedding the light on a further advantage of SMB-sheet therapy in safety over the current method, in addition to the elimination of arrhythmogenicity.

3.5. Improved cardiac performance by SMB-sheet therapy

Previous clinical trials of intramyocardial injection of SMB suspension showed insufficient or inconsistent therapeutic effects, in corresponding with poor donor cell survival/engraftment [1,2]. Considering the ability to increase donor cell survival (Fig. 2A), the use of the cell-sheet technique may solve this issue. Indeed, echocardiography showed that LV ejection fraction was improved in the IM group compared to the Cont group, and this was further enhanced in the Sheet group at day 28 post treatment (Fig. 5A). In addition, both LV end-diastolic and end-systolic dimensions in the Sheet group were smaller than those in other groups (Fig. 5B, C). Cardiac catheterization also showed improved cardiac function in the Sheet group compared to the other groups (Fig. 5E). LV end-diastolic

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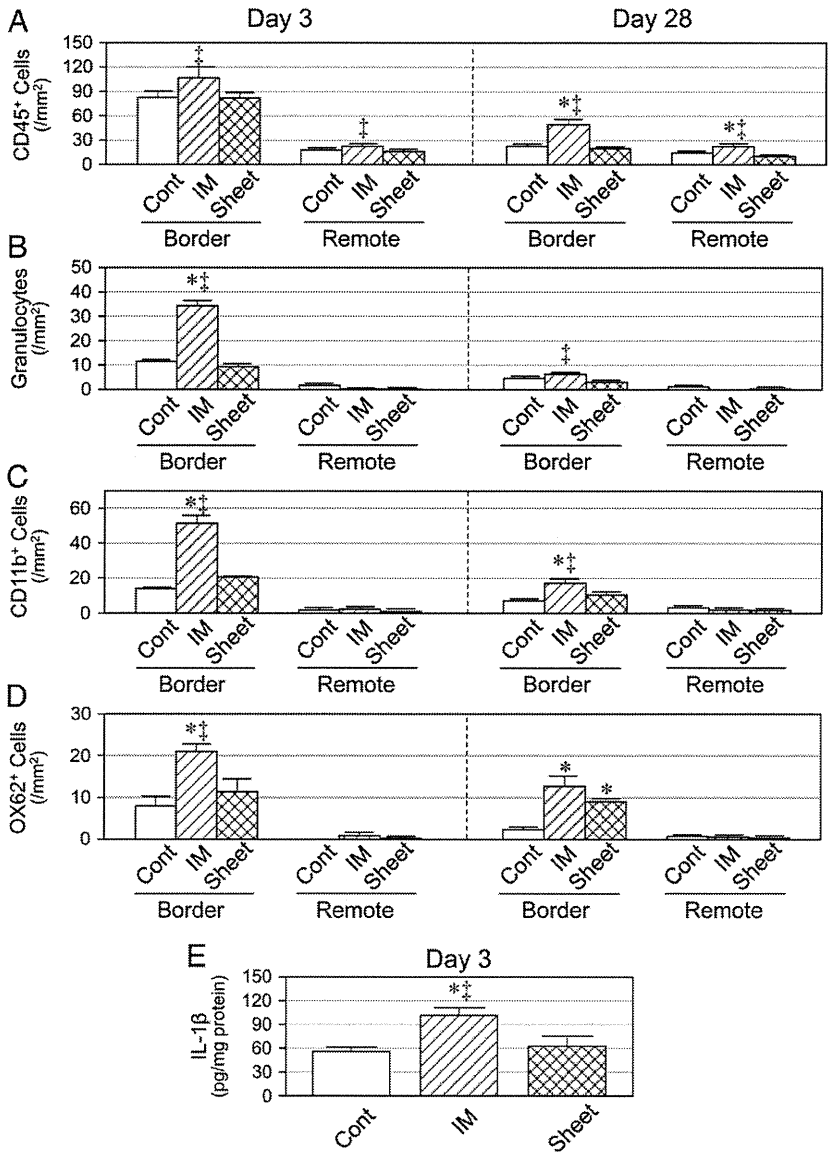

Fig. 3. Prevention of exacerbation of myocardial inflammation by SMB-sheet therapy. Immunolabeling demonstrated that the number of $CD45^+$ cells accumulating in the myocardium was persistently increased in the IM group, but not in the Sheet group, widely in both border and remote areas (A). In addition, in the IM group, there was increased accumulation of granulocytes (B), $CD11b^+$ monocytes/macrophages (C), and $OX62^+$ dendritic cells (D) at both day 3 and 28. ELISA showed increased myocardial IL-1β levels at day 3 in the IM group compared to other groups (E). $^*p<0.05$ vs. Cont group, $^‡p<0.05$ vs. Sheet group, n = 4–6 in each group.

pressure was lower and developed pressure was higher in the Sheet group compared to the Cont group.

3.6. Mechanisms underpinning the therapeutic effects of SMB-sheet therapy

We subsequently studied the mechanism by which SMB-sheet therapy improved cardiac function. Agreeing with previous findings [1,5], we could not find any cardiomyocyte-like cells derived from donor SMBs (via either trans-differentiation or fusion) in any group by histological trafficking of CM-DiI labeled SMBs. Instead, our histological studies detected favorable changes in each cardiac component in the Sheet group. These included reduced infarct size (Fig. 5D), decreased extracellular collagen deposition (Fig. 6A–G), increased vascular formation (Fig. 6H–N), and attenuated cardiomyocyte hypertrophy (Fig. 6O–R) at day 28 in the Sheet group compared o the Cont group. Of note, these beneficial effects were widely observed not only in the border areas but also in the remote areas. These beneficial changes were also seen in the IM group, but to a reduced extent in general, correlated with the reduced donor SMB presence.

To obtain a further mechanistic insight of the therapeutic effects of SMB-sheet therapy, we analyzed myocardial expression of reportedly relevant genes by quantitative RT-PCR. As a result, we observed that expression of *IL-10*, *HIF1-α*, *MMP-2*, *TIMP-1*, *IGF-1*, and *SDF-1*, was significantly upregulated in the Sheet group at day 3 compared to the Cont group (Fig. 7). These data corresponded well with the above histological findings of beneficial changes in cardiac components as the paracrine effects. An anti-inflammatory cytokine, *IL-10*, is known to increase donor cell survival after cell transplantation and also increase angiogenesis [19]. Upregulation of a major pro-angiogenic factor, *HIF1-α* [20], could also play a role in the increased capillary density in the Sheet group, while upregulation in *MMP-2* and *TIMP-1* would correlate with the reduced fibrosis [3]. Upregulation of *IGF-1* and *SDF-1*, which are known to stimulate endogenous progenitor cells [21,22], might have increased endogenous regeneration. Of note, the upregulation of these genes in the Sheet group was mostly reduced to the post-MI background level

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A

	Lung	Liver	Spleen	Kidney
IM	4/4 (100%)	0/4 (0%)	1/4 (25%)	1/4 (25%)
Sheet	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
p value	0.0047	NS	NS	NS

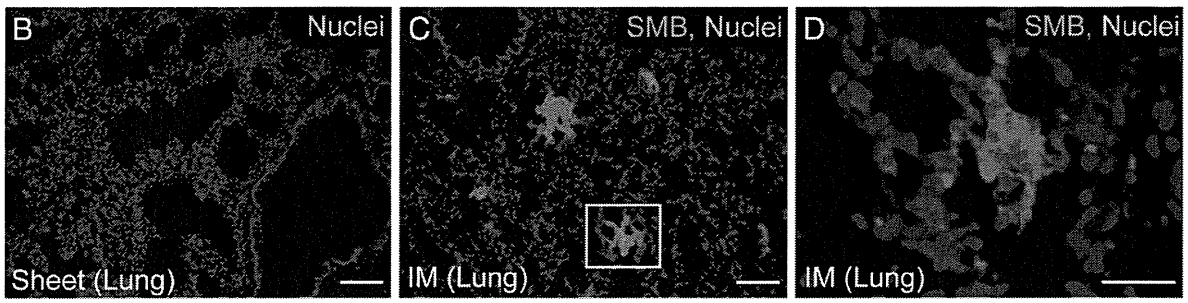


Fig. 4. Attenuated ectopic donor cells in other organs after SMB-sheet therapy. At day 28 post treatment, PCR for *sry* detected the presence of donor cells in the lungs of all host animals in the IM groups (A). There were no donor cells detected in any organs after SMB-sheet therapy. Fluorescence observation demonstrated that SMBs (CM-DiI-labeled; orange) were widely distributed in the lung, forming discrete loci, at day 28 in the IM group (C, D), while there were no SMBs detected in the lung of the Sheet group (B). Blue (DAPI) represents nuclei. Scale bar = 100 μm in (B, C) and 30 μm in (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

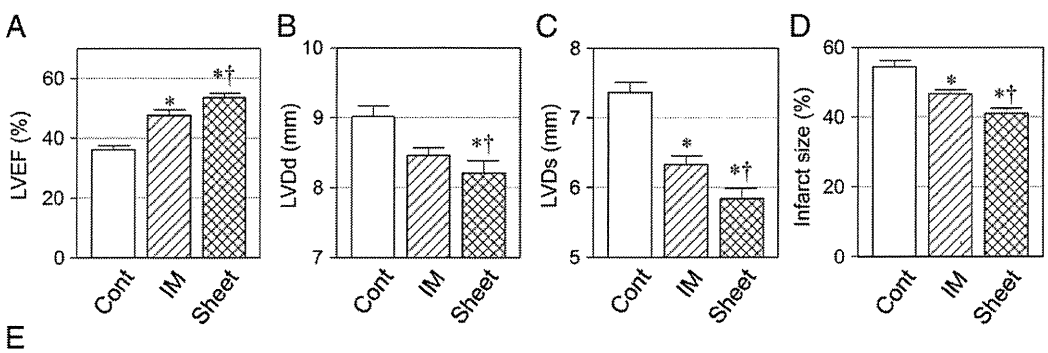
in the Cont group by day 28, in corresponding with the reduced donor cell presence by day 28 (Fig. 2A). Despite this, cardiac function remained improved for at least 28 days after SMB-sheet therapy compared to the sham control. This let us speculate that paracrine mediators would contribute to the myocardial recovery mainly during the early phase after the treatment, and the effects on cardiac function and structure, once established, could last for a longer time.

4. Discussion

This pre-clinical study demonstrated that epicardial placement of SMB-sheets, which were generated with temperature-responsive culture dishes without enzymatic treatment and without artificial scaffolds, overcame both key problems of the current SMB transplantation method: critical arrhythmogenicity and limited therapeutic efficacy [1,2].

This study for the first time provided comprehensive, quantitative evidence showing that the use of the cell-sheet technique prevents arrhythmogenicity associated with SMB-transplantation therapy to the heart. We established a rat model in which intramyocardial SMB injection induced frequent ventricular arrhythmias, including VT, in an equivalent manner to the previous clinical reports [1,2,23]. Using this clinically-relevant model, our continuous monitoring using radio-telemetry clearly demonstrated that arrhythmia occurrence was reduced after SMB-sheet therapy to the base line (MI hearts; Cont group). Together with another novel finding that SMB-sheet therapy attenuated ectopic donor cell distribution in the lung, which was seen after intramyocardial injection, these data highlight the improved safety of SMB-sheet therapy.

Furthermore, our results also imply information regarding the mechanism underlying SMB-induced arrhythmias, which remains uncertain [23]. Previous *in vitro* studies have suggested a possible role of automaticity or spontaneous electrical activity of the SMBs on the



E

	HR (bpm)	LVEDP (mmHg)	LVDP (mmHg)	Max dP/dt (mmHg/s)	Min dP/dt (mmHg/s)	Contractility Index
Cont	375±8	18.1±1.2	79.7±1.8	6832±138	-5686±351	105.9±3.6
IM	376±9	12.8±0.9*	95.7±3.1*	7598±277*	-6764±413	119.0±4.1*
Sheet	368±7	10.8±1.2*	105.4±4.3*†	8707±311*†	-7558±350*	124.1±5.3*

Fig. 5. Improved cardiac performance by SMB-sheet therapy. Enhanced cardiac function, reduced heart dimensions and improved hemodynamics in the Sheet group, compared to other groups, were detected by using echocardiography (A–C) and catheterization (E) at day 28. Picrosirius red staining showed the smallest infarct size in the Sheet group (D). HR = heart rate, LVDd = LV end-diastolic dimension, LVDs = LV end-systolic dimension, LVEDP = LV end-diastolic pressure, LVDP = LV developed pressure, *$p<0.05$ vs. Cont group, †$p<0.05$ vs. IM group, n = 10–11 in each group in (A–C), 4–5 in (D), and 7–8 in (E).

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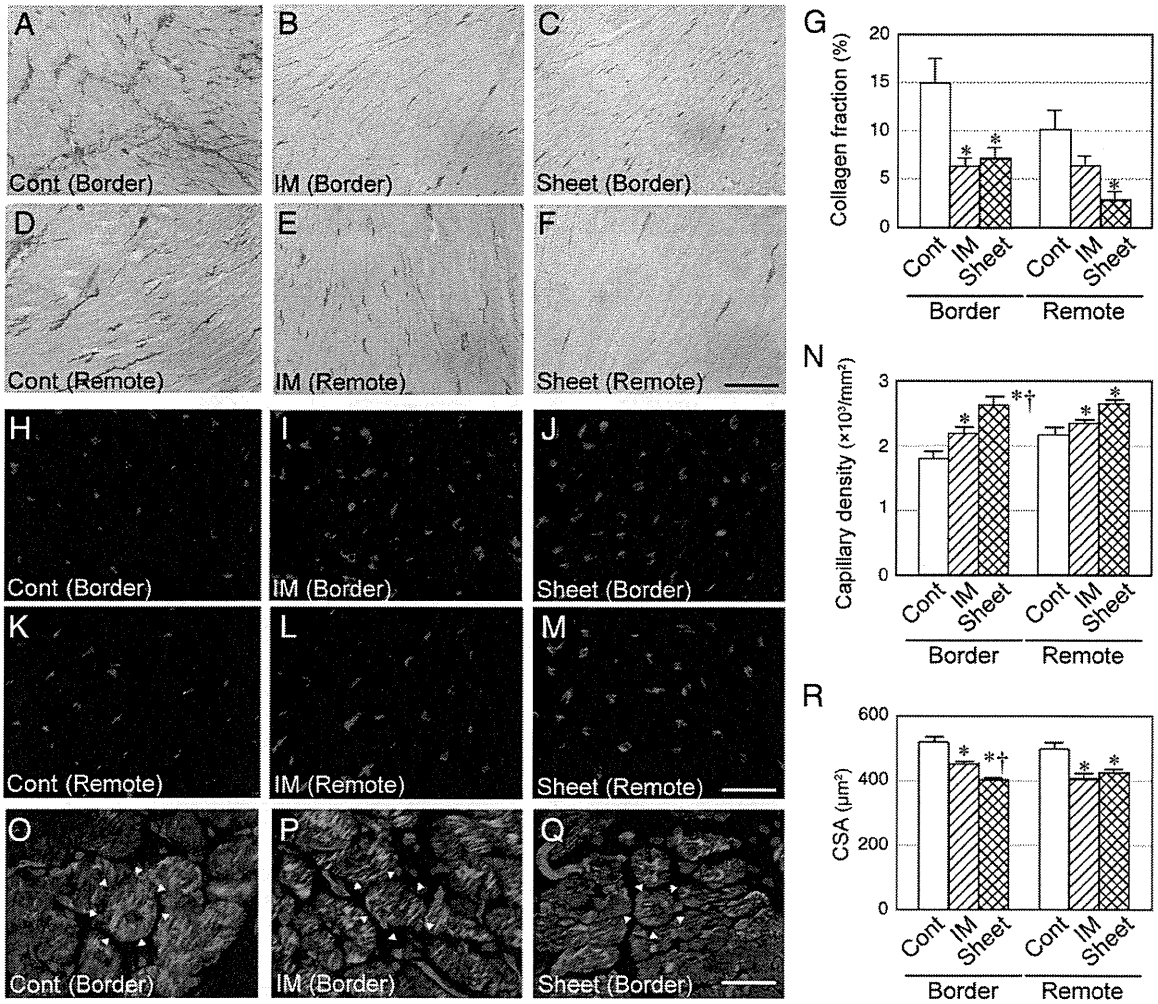

Fig. 6. Recovery of post-MI failing myocardium by SMB-sheet therapy. Picrosirius red staining showed that extracellular collagen deposition (red color) in the Cont group (A, D) was reduced in the IM (B, E) and Sheet groups (C, F) at day 28. Semi-quantified collagen volume fraction is shown in (G). Isolectin B_4 staining demonstrated that capillary density (red color) at day 28 was increased in the IM (I, L) and Sheet groups (J, M) compared to the Cont group (H, K). Capillary density was quantified as the number of capillary vessels per mm^2 (N). Cardiomyocyte size (CSA = cross sectional area) was reduced in the Sheet group compared to the Cont group (O–R). Green signals for cardiomyocytes (cTnT); red for endothelial cells (Isolectin B4). Cells surrounded by white arrowheads represent the cardiomyocytes chosen for the CSA measurement. Scale bar = 100 μm in (A–F) and 30 μm in (H–M, O–Q). $^{*}p<0.05$ vs. Cont group, $^{\dagger}p<0.05$ vs. IM group, n = 4–6 in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

arrhythmogenicity [24,25]; however, results of our *in vivo* study were rather negative for these, as the cell-sheet technique showed reduced arrhythmogenicity despite the increase in surviving SMBs. Alternatively, our results propose that the formation of local heterogeneity by intramyocardial injection may play a causative role [4,5,23]. We observed that intramyocardial injection formed discrete cell-clusters composed of donor SMBs and host inflammatory cells. Given our histological finding that grafted SMBs rarely formed gap junctions with host cardiomyocytes, such intramyocardial heterogeneity would cause physical disturbance to electrical impulse propagation, resulting in conduction delay, block, and eventually re-entrance arrhythmias. This concept was supported by additional finding of the elongated QRS duration after intramyocardial SMB injection. In contrast, after SMB-sheet therapy, the majority of SMBs remained on the epicardial surface, obviating the intramyocardial tissue disruption. This resulted in the maintenance of electrical conductance, therefore preventing arrhythmogenicity. In addition, this study provided new data which suggest another mechanism by which intramyocardial SMB injection induced arrhythmias; exacerbation of post-MI arrhythmogenic substrates [13,23]. Intramyocardial SMB injection resulted in persistent and widespread increase of inflammatory response, which could amplify the pathological substrates and increase arrhythmia susceptibility. In contrast, SMB-sheet therapy did not aggravate myocardial inflammation nor therefore increase arrhythmogenic substrates.

Insufficient therapeutic efficacy was another key issue of SMB transplantation [1,2], and this may also be solved by the use of the cell-sheet technique. It has been shown that SMB-sheet therapy improves performance of the damaged heart in various models [8–10], but this study further confirms this ability of SMB-sheet therapy in a more comprehensive manner by direct comparison with intramyocardial injection. In addition, we showed that this augmented efficacy was correlated to the increased donor cell engraftment. Our quantitative assessment showed that donor cell survival at day 3 was 3.5-fold higher in SMB-sheet therapy compared to intramyocardial injection, suggesting that the use of the cell-sheet technique increased the early retention and/or survival of donor cells. Furthermore, the surviving donor cell number at day 28 was more apparent, 9.2-fold, larger in SMB-sheet therapy. This indicates that SMBs grafted by the cell-sheet technique survived between day 3 and 28 with a higher rate (17.5/52.3 = 0.33) compared to intramyocardial injection (1.9/15.0 = 0.13), suggesting that the cell-sheets would provide a more comfortable environment for SMBs to survive.

Consistent to previous reports [1,5], we did not find donor SMB-derived cardiomyocytes either after SMB-sheet therapy or intramyocardial SMB

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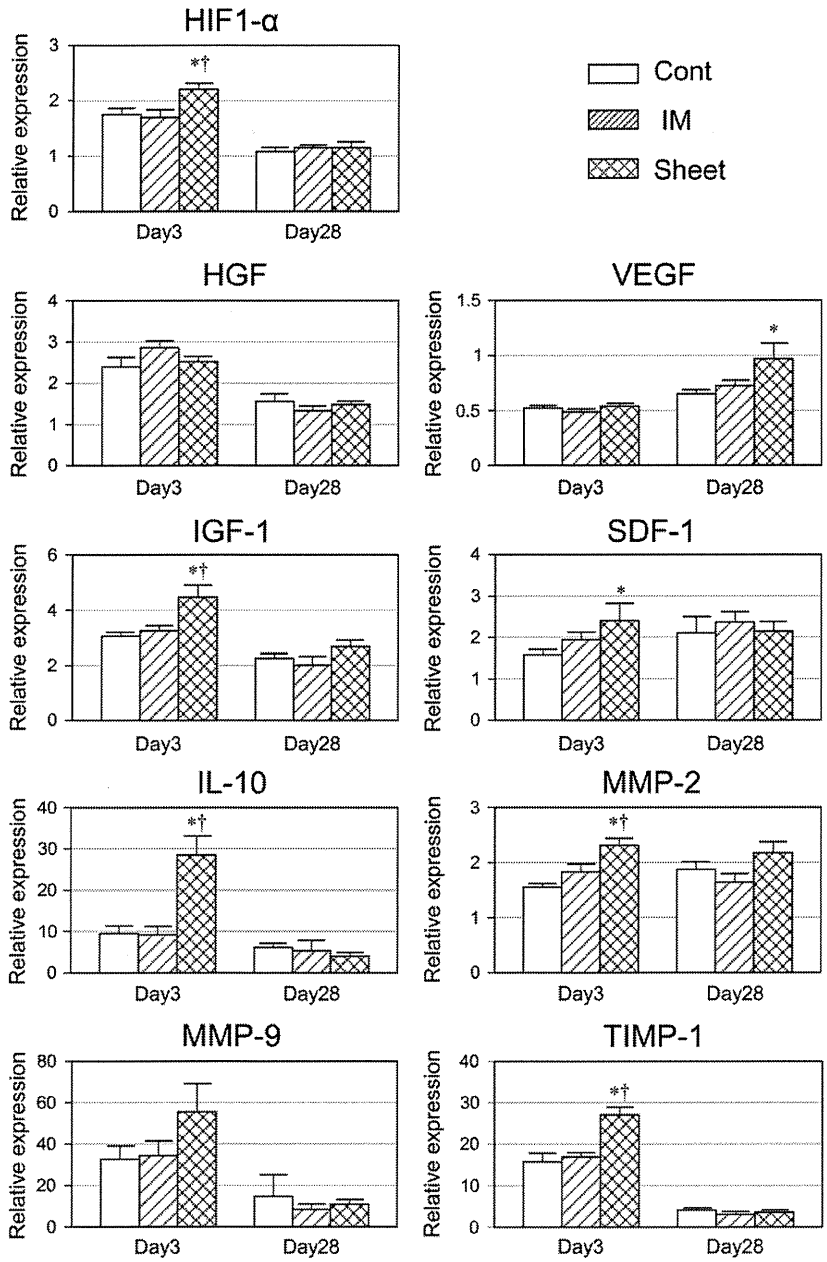

Fig. 7. Myocardial gene expression after SMB-sheet therapy. Quantitative RT-PCR detected upregulation of multiple genes in the Sheet group at day 3, though this was largely diminished by day 28. Expression levels were normalized to that in normal hearts (n = 3), which was assigned a value of 1.0. *p<0.05 vs. Cont group, †p<0.05 vs. IM group, n = 4–6 in each group.

injection. Considering the beneficial histological changes, including decreased infarct size, reduced fibrosis, increased neovascular formation, and attenuation of cardiomyocyte hypertrophy, the "paracrine effect" may be the major mechanism responsible for the therapeutic benefits by SMB-sheet therapy. As regards this, we found that a group of possibly relevant molecules, including *IL-10*, *HIF1-α*, *MMP-2*, *TIMP-1*, *IGF-1* and *SDF-1*, were upregulated in the heart after SMB-sheet therapy. We could not specify what paracrine molecules among these (or others) were really responsible for the effects of SMB-sheet therapy, but speculate that the obtained effects may be a result of the net input of certain groups of paracrine molecules, rather than a single molecule. However, further investigation is needed to conclude this issue.

In conclusion, SMB-sheet therapy attenuated both the arrhythmogenicity and ectopic donor cell distribution in the lung that were associated with the current method for SMB transplantation, together with augmented therapeutic efficacy in the treatment of heart failure. These data may encourage the development of this emerging approach towards clinical application.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Long-Term Follow-Up Evaluation of Results From Clinical Trial Using Hepatocyte Growth Factor Gene to Treat Severe Peripheral Arterial Disease

Hirofumi Makino, Motokuni Aoki, Naotaka Hashiya, Keita Yamasaki, Junya Azuma, Yoshiki Sawa, Yasufumi Kaneda, Toshio Ogihara and Ryuichi Morishita

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Hirofumi Makino, Motokuni Aoki, Naotaka Hashiya, Keita Yamasaki, Junya Azuma, Yoshiki Sawa, Yasufumi Kaneda, Toshio Ogihara, Ryuichi Morishita

Objective—As angiogenic growth factors can stimulate the development of collateral arteries, a concept called therapeutic angiogenesis, we performed a phase I/IIa open-label clinical trial using intramuscular injection of naked plasmid DNA encoding hepatocyte growth factor (HGF). We reported long-term evaluation of 2 years after HGF gene therapy in 22 patients with severe peripheral arterial disease.

Methods and Results—Twenty-two patients with peripheral arterial disease or Buerger disease staged by Fontaine IIb (n=7), III (n=4), and IV (n=11) were treated with HGF plasmid, either 2 mg or 4 mg ×2. Increase in ankle-branchial pressure index >0.1 was observed in 11 of 14 patients (79 %) at 2 years after gene therapy and in 11 of the 17 patients (65%) at 2 months. Reduction in rest pain (>2 cm in visual analog scale) was observed in 9 of 9 patients (100%) at 2 years and in 8 of 13 (62%) patients at 2 months. At 2 years, 9 of 10 (90%) ischemic ulcers reduced by >25%, accompanied by a reduction in the size of ulcer. Severe complications and adverse effects caused by gene transfer were not detected in any patient throughout the period up to 2 years.

Conclusion—Overall, the present study demonstrated long-term efficacy of HGF gene therapy up to 2 years. These findings may be cautiously interpreted to indicate that intramuscular injection of naked HGF plasmid is safe, feasible, and can achieve successful improvement of ischemic limbs as sole therapy. **(*Arterioscler Thromb Vasc Biol.* 2012;32:2503-2509.)**

Key Words: angiogenesis ■ peripheral arterial disease ■ plasmid DNA ■ hepatocyte growth factor ■ gene therapy

The clinical consequences of peripheral arterial disease (PAD) include pain on walking (claudication), pain at rest, and loss of tissue integrity in the distal ischemic limbs. Recent progress in molecular biology has led to the development of gene therapy as a new strategy to treat a variety of cardiovascular diseases using angiogenic growth factors, such as vascular endothelial growth factor (VEGF).[1–3] However, recent reports have documented the disadvantage of VEGF, such as edema formation,[1,2,4] and recent studies have demonstrated that VEGF121 did not show improvement in clinical end points.[5] In contrast, although phase II nonviral fibroblast growth factor-1 clinical trial revealed a significant decrease in the amputation rate,[6] large-scale phase III trial failed to show its efficacy.[7]

Our previous reports demonstrated that intramuscular gene transfer of naked plasmid DNA containing the sequence encoding human hepatocyte growth factor (HGF), a potent angiogenic growth factor,[8–15] in patients with critical limb ischemia (CLI) resulted in a significant increase in ankle-branchial pressure index (ABI) and improved clinical symptoms, such as ischemic ulcer at 2 months after transfection.[16,17] In addition, 3 randomized placebo control studies (1 phase III trial in Japan and 2 phase II trials in the United States) demonstrated the healing of ulcer, decrease in rest pain, increase in transcutaneous oxygen tension, or toe pressure at 2 or 6 months after the treatment.[18–20] These clinical trials including ours revealed clinical therapeutic values of HGF gene therapy to treat CLI. Of particular interests, different from VEGF, HGF gene therapy did not show edema as side effects. However, previous studies only showed short-term efficacy of HGF gene therapy but not long-term efficacy, amputation rate, and mortality. Thus, we sought to evaluate the long-term safety and clinical effects of direct intramuscular gene transfer of naked human HGF plasmid DNA on ABI, ulcer, and rest pain as functional end points, and amputation and death rate as hard end points up to 2 years after gene therapy in 22 no-option patients with severe PAD refractory to maximal medical therapy and not amenable to conventional revascularization. The present study documented the continuous improvement in clinical symptoms, such as the healing of ulcers. Despite

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this limitation of the open-label trial, we demonstrated that intramuscular injection of naked HGF plasmid might decrease amputation rate and mortality up to 2 years after gene therapy compared with historical data.

Materials and Methods

Clinical Trial Design

This study was conducted as a phase I/IIa investigator-initiated clinical trial, as previously described.[16,17] Part of the information included in this article has been published in the previous reports.[16,17] Briefly, the pVAX1 plasmid vector (Invitrogen Corporation, Carlsbad, CA) was selected for HGF construct. To minimize the possibility of chromosomal integration, insertional mutagenesis through the activation of oncogenes, or inactivation of tumor suppressor genes, all sequences with possible homology to the human genome have been removed from the pVAX1 DNA, along with any sequence not necessary for replication in *Escherichia coli* or for expression of recombinant protein in mammalian cells. The pVAX1 HGF plasmid consists of a cDNA fragment of human HGF inserted into the pVAX1 vector.

In this single-center prospective, open-label study, patients were enrolled if they (1) had chronic CLI, including rest pain and a nonhealing ischemic ulcer, or had severe intermittent claudication with maximum walking distance <200 m for a minimum of 4 weeks; (2) had been resistant to conventional drug therapy for >4 weeks; (3) were not candidates for surgical or percutaneous revascularization based on usual standards of practice; (4) did not have previous or current neoplasms; and (5) did not have severe retinopathy, as documented previously.[16,17] Objective documentation of ischemia, including resting ABI of >0.6 in the affected limb on 2 consecutive examinations performed 1 week apart, was necessary. Patients were observed for 4 weeks under conventional drug therapy to confirm that their clinical symptoms and objective parameters were not improved. The study was approved by the Ministry of Health, Labor, and Welfare, and Ministry of Education, Culture, Sports, Science, and Technology. Twenty-two limbs of 17 patients (13 men and 4 women; 59.6±12.0 years) with arteriosclerosis obliterans (ASO; n=14) or Buerger disease (thromboangitis obliterans [TAO]; n=8) staged as Fontaine IIb, III, or IV underwent direct intramuscular gene transfer of naked plasmid DNA encoding HGF. The characteristics of the patients are shown in Table 1 and in a previous article.[17] In 4 patients, plasmid was administrated to bilateral legs one-by-one at an interval >3 months. One patient was treated bilaterally at the same time.

Table 1. Patient Characteristics

Case No.	Disease	Age, y	Sex	Fontaine Stage	Injection Dose, mg	Background Factors
1	ASO	56	M	III	2	HT and current smoking
2	ASO	42	M	IV	2	DM, HT, and CRF on HD
3	TAO	47	M	IV	2	past smoking
4	TAO	64	F	III	2	—
5	TAO	66	M	IV	2	HT
6	ASO	69	M	IV	2	DM, HT, and HL
7	TAO	67	M	IV	4	HT
8	TAO	61	M	III	4	HT, HL, and past smoking
9	ASO	66	M	IIb	2	DM, HT, and CRF on HD
10	ASO	60	M	IIb	4	DM, HT, and HL
11	ASO	66	M	IIb	4	DM, HT, and HL
12	ASO	70	F	IV	2	DM and HL
13	TAO	59	M	IIb	2	HL and past smoking
14	ASO	78	F	III	2	HT
15	TAO	53	M	IV	2	past smoking
16	ASO	66	M	IIb	4	DM, HT, and CRF on HD
17	TAO	27	F	IV	2	past smoking
18	ASO	66	M	IIb	2	DM, HT, and HL
19	ASO	66	M	IV	4	HT, HL, DM, and past smoking
20	ASO	63	M	IV	4	DM, HT, and CRF on HD
21	ASO	63	M	IV	2	DM, HT, and CRF on HD
22	ASO	66	M	IIb	4	HT, HL, DM, and past smoking

ASO indicates arteriosclerosis obliterans; TAO, thromboangitis obliterans (Buerger disease); M, male; F, female; HT, hypertension; DM, diabetes mellitus; CRF, chronic renal failure; HD, hemodialysis; HL, hyperlipidemia.

Case Nos. 5 and 7, 9 and 16 , 11 and 18, and 19 and 22 represent same patients, respectively. In those 4 patients, plasmid was administrated to bilateral legs one-by-one at an interval >3 months. Case No. 20 and 21 also represent the same patient, who was treated bilaterally at the same time.

Intramuscular Injection of Naked Plasmid DNA Encoding Human HGF

Each patient received an intramuscular injection of naked plasmid HGF DNA, as described previously.[16,17] This study was divided into 2 stages. As stage 1, for the initial 6 patients with Fontaine stage III or IV, a test intramuscular injection of a small dose (test injection) (0.4 mg plasmid DNA) was performed to examine acute or subacute allergy to plasmid DNA. After confirmation of no allergic reaction or anaphylaxis, 2 mg of naked HGF plasmid DNA was intramuscularly injected 2 weeks after test injection into the calf or distal thigh muscles of the ischemic limb by direct intramuscular injection under ultrasonic guidance. The injection sites of plasmid DNA are described in our previous preliminary report.[17] Four weeks after the initial injection, a second injection (2 mg) was similarly administered, giving a total dose of 4 mg plasmid DNA per patient. Results of this study up to 6 months were previously published.[17] As stage 2, 16 limbs of 12 patients with Fontaine stage IIb, III, or IV were intramuscularly injected with a therapeutic dose (2 mg/4 sites or 4 mg/8 sites) of naked HGF plasmid DNA. The doses of HGF plasmid were randomly allocated using an envelope method. HGF plasmid DNA (0.5 mg) was diluted in sterile saline solution up to 3 mL, and 4 or 8 aliquots (total 2 or 4 mg/12 or 24 mL) were administered as stage 1. Four or 8 injection sites were selected arbitrarily, according to the angiographic findings and the available muscle mass. Four weeks after the initial injection, the second injection was similarly administered, giving a total dose of 4 or 8 mg plasmid DNA per patient.

Patient Follow-Up and Assessment

Briefly, patients were followed by physical examination (including change in ischemic ulcers), blood analysis, visual analog scale pain scale (VAS), and measurements of ABI weekly during the first 12 weeks, every other week for the next 8 weeks, monthly for the next 12 weeks, and then every 3 months up to 2 years after the first injection of plasmid DNA. ABI was measured using plethysmographic wave or Doppler wave system, IMEX (Getz Bros. Co). Maximum walking distance was measured at baseline, 1, 2, 3, and 24 months using treadmill at 2.4 km/h with 5% incline. During the study period, the development of malignant tumors and progression of retinopathy were carefully investigated by various examinations.

Statistical Analysis

All values are expressed as mean±SD. To compare the clinical parameters (ABI, ulcer size, VAS, and maximum walking distance) between baseline and each time point, the Dunnet test was used. To test the long-term increasing or decreasing tendency of these clinical parameters, the regression coefficient of the time course was calculated. To assess the time-dependent change in these parameters

between 2 groups, such as ASO and TAO, or CLI and intermittent claudication, the regression coefficient of time course was compared using ANCOVA. $P<0.05$ was considered statistically significant. All tests were 2-tailed. To avoid any bias, an evaluation committee independent from the trial investigators checked all the data. The JMP statistical software was used for calculations.

Results

Long-Term Efficacy of HGF Gene Therapy

In this study, we evaluated ABI as a functional end point and resting pain, as assessed by VAS or the size of ischemic ulcer, as a clinical end point. As previously reported,[17] ABI significantly increased from 0.46±0.08 (n=17) at baseline (before administration) to 0.59±0.13 (P=0.0135; n=17) at 8 weeks after injection (Figure 1A). Even at 2 years after gene therapy, ABI still increased significantly (0.61±0.13; P=0.0053; n=14), whereas the peak increase in ABI was observed at 6 months after treatment (0.65±0.15). There was no significant time dependency in ABI increase (regression coefficient, 0.00359; P=0.051). We also analyzed the results of ABI according to Fontaine stage III–IV (CLI) and stage II (intermittent claudication). There was no significant difference in the improvement of ABI between the 2 groups (P=0.1620) (Figure I in the online-only Data Supplement). When an increase in ABI of >0.1 was assumed to be an improvement, according to the standard of Rutherford, 11 of 17 patients (65%) showed a positive response at 2 months after transfection (Table 2). The efficacy rate of ABI after 2 years reached 79% (11 of 14 patients). These results demonstrated that functional improvement in blood flow induced by HGF still continued up to 2 years after transfection, despite the short duration of gene expression.

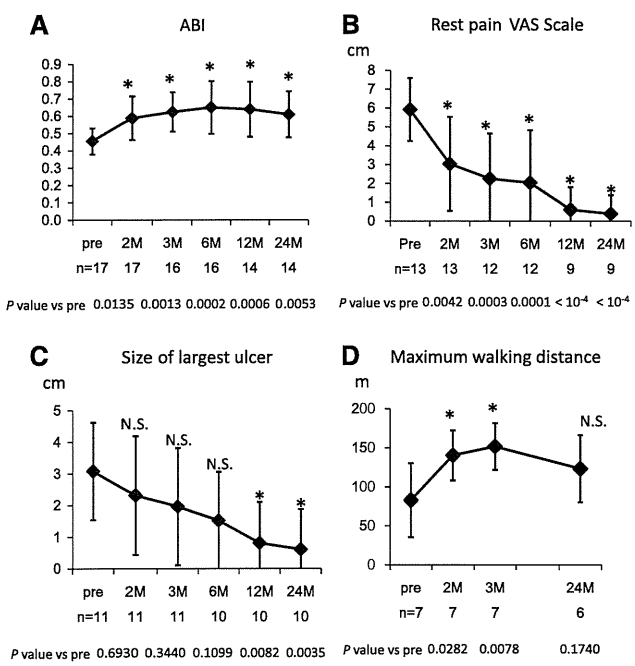

Figure 1. Serial changes in parameters. **A**, Changes in ankle-branchial pressure index (ABI). **B**, Sizes of largest ulcers. **C**, Rest pain assessed with visual analog scalepain scale (VAS). **D**, Maximum walking distance. Data are expressed as mean±SD. For comparison between baseline and each time point, data were analyzed using Dunnett test. Asterisks indicate statistically significant. Pre indicates baseline; 2M, 6M, 12M, and 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection; N.S., not significant.

Table 2. Serial Changes in Efficacy Rate

	2M	6M	12M	24M
ABI (increase >0.1)	11/17 (65)	13/16 (81)	10/14 (71)	11/14 (79)
Rest pain (reduction >2 cm)	8/13 (62)	9/12 (75)	9/9 (100)	9/9 (100)
Largest ulcer size (reduction >25%)	7/11 (64)	8/10 (80)	9/10 (90)	9/10 (90)

ABI indicates ankle-branchial pressure index.

Efficacy rates are indicated as effective cases/total cases (%). An ABI increase by >0.1, a visual analog scalepain scale reduction by >2 cm, and an ulcer diameter reduction by >25% were defined as effective cases. 2M, 6M, 12M, 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection.

To evaluate the effects of HGF gene therapy on clinical symptoms, we evaluated rest pain using visual analog scale, as a standard method for evaluation of pain. As shown in Figure 1B, rest pain had significantly improved at 2 months after transfection (P=0.0042). Importantly, rest pain gradually improved in a time-dependent manner (regression coefficient, −0.184; $P<0.0001$). Rest pain reduced from 5.92±1.67 (n=13) at baseline to 0.39±0.99 (n=9) at 2 years after transfection. In this trial, 9 of 9 patients (100%) demonstrated improvement in rest pain over 2 cm at 2 years after transfection and in 8 of 13 patients (62%) at 2 months after transfection (Table 2). In this trial, a total of 25 ischemic ulcers were found in 11 patients at baseline. The size of the largest ulcer had significantly reduced from 3.08±1.53 (n=11) at baseline to 0.61±1.27 (n=10) at 2 years (Figure 1C). Consistent with the gradual improvement in rest pain, ischemic ulcer also significantly reduced in a time-dependent manner (regression coefficient, −0.0926; P=0.0004; Figure 1C). At 2 years after transfection, the reduction in ischemic ulcer reached 0.61±1.27 (n=10). Considering an improvement in ischemic ulcers by >25% to be evaluated as positive, 18 of 25 ulcers (72%) had improved at 2 months after transfection. It is noteworthy that at 2 years after transfection, 17 of 18 ulcers (94%) reduced by >25%. As shown in Figure 1C, the largest ischemic ulcer diameter also reduced after transfection. Seven of 11 patients (efficacy rate 64%) demonstrated an improvement in the largest ischemic ulcer diameter by >25% at 2 months and 9 of 10 patients (efficacy rate 90%) at 2 years (Table 2). At 2 months, one of 11 ulcers completely healed. Typical examples of the changes in ischemic ulcers in patients with ASO and Buerger disease are shown in Figure 2. Two years after transfection, 7 of the 11 patients with ulcers achieved complete healing (Table 3). In this trial, 7 patients with Fontaine IIb stage were also tested. Peak walking time had significantly increased at 2 and 3 months after second transfection (Figure 1D); however, this effect was diminished at 2 years. In addition, we compared the time-dependent changes in patients with ASO and TAO, because these 2 groups have different characteristics in pathophysiology and clinical course. Both groups showed an improvement in ABI, ulcer size, and VAS (Figure II in the online-only Data Supplement). However, there was no significant difference in the tendency between the 2 groups. We also evaluated the transition of the Fontaine stage. As shown in Table 4, 5 of 11 patients with

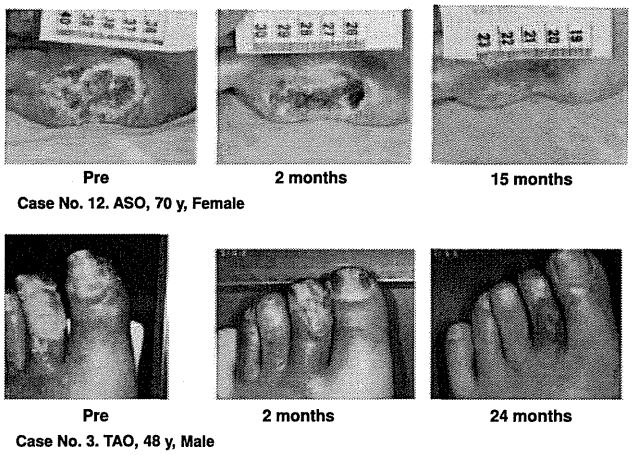

Figure 2. Typical examples of change in ischemic ulcers in patients. The ischemic ulcer in case no.12 was completely healed in 15 months after the second injection. Case no. 3 healed in 24 months. 2 months, 15 months, 24 months indicates 2, 15, and 24 months, respectively, after the second injection. Pre indicates before injection; TAO, thromboangitis obliterans.

stage IV improved to stage IIb at 2 years. Similarly, 1 of 4 patients with stage III improved to stage IIb.

Safety Evaluation

We also evaluated the safety of HGF gene therapy up to 2 years. Especially, we focused on the incidence of angiogenesis-related disease, such as tumor, and other severe complications. The initial trial up to 6 months documented no evidence of the allergic reaction related to plasmid DNA.[17] Similarly, 2-year follow-up also documented that none of the patients showed an allergic or anaphylactic reaction. No serious adverse events related to gene therapy were observed from the initial analysis up to 2 years after transfection (Table 5). To date, development of tumors or progression of diabetic retinopathy has not been observed in any patient transfected with HGF plasmid DNA during the trial up to 2 years after transfection. No patient had a major amputation during the 2 years, whereas 1 patient (No. 17) had a minor amputation. In addition, 2 patients died because of hyperkalemia after operation at 7 months and pneumonia at 7 months, respectively (Table 5). The committee independent from the investigators evaluated these incidents as not related to HGF gene therapy. The cumulative rates of major amputation and mortality are shown in Table 6.

Discussion

The present study reported 2-year follow-up study of HGF gene therapy in 22 cases. The initial end points evaluated at 2 months after transfection exhibited a significant improvement in functional end point as assessed by ABI measurement and clinical end points as assessed by VAS and ischemic ulcer.[17] After the initial analysis, we performed the long-term analysis up to 2 years after transfection in this study. The most striking point from the present study is that an improvement in ABI, ulcer size, and rest pain continued up to 2 years after transfection. It is well known that the duration of gene expression driven by naked plasmid DNA is usually not longer than 1 month after injection. Indeed, our preclinical study also confirmed the transient expression of transgene using naked plasmid HGF DNA.[10,13,14] Nevertheless, the present study demonstrated that the efficacy of HGF gene therapy was sustained up to 2 years after transfection. Especially, ABI as a functional end point to reflect an improvement in blood flow was significantly increased up to 2 years after transfection. To our knowledge, such an improvement has not previously been achieved spontaneously or with medical therapy in patients with CLI. One of the possible explanations is that the increase in blood vessels induced by HGF at early time point might enlarge the collateral vessels at later time point over the transient expression of exogenous HGF. Alternatively, as exogenous HGF upregulated endogenous HGF and VEGF through an essential transcription factor for angiogenesis, ets-1,[11,21] increase in endogenous HGF and VEGF might work for the continuous growth of collateral formation. Moreover, the lymphangio genetic effects of HGF might contribute the reduction in edematous lesion in Buerger disease.[22] For example, the fingers of case 3 patients were edematous at baseline and 2 months, whereas his edema almost disappeared at 24 months after the therapy (Figure 2, lower). However, 2 years after transfection, ABI seems to be slightly decreased compared with 6 months. Another treatment using HGF gene might be necessary to develop and maintain the collateral formation, although further studies must be done.

It is more important to consider the clinical application that clinical symptoms, such as rest pain and ischemic ulcer, were gradually decreased in a time-dependent manner. The improvement in these clinical symptoms was different from the time course of ABI. ABI and maximum walking distance peaked at around 3 to 6 months and then decreased a little, whereas rest pain (VAS scale) and ulcer size improved continuously until 2 years after gene therapy. Possible explanation for this discrepancy includes that improvement in blood flow by HGF gene therapy might increase the local flow, resulting in the continuous improvement in resting pain and ulcer size. In patients with CLI, it is well known that critical point to improve resting pain and ulcer would have existed. Probably, the increase in ABI over the critical value 0.5 might lead to the

Table 3. Healing of Ulcers

	1M	2M	3M	6M	12M	24M
Largest ulcers	0/11 (0)	1/11 (9)	2/11 (18)	4/10 (40)	6/10 (60)	7/10 (70)
All ulcers	7/25 (28)	7/25 (28)	9/25 (36)	10/21 (48)	16/21 (76)	18/21 (86)
Complete healing	0/11 (0)	1/11 (9)	1/11 (9)	4/10 (40)	6/10 (60)	7/10 (70)

Largest ulcers and all ulcers are indicated as number of healed ulcers/number of ulcers at baseline (%). Complete healing shows the number of patients whose ulcers were completely healed. One patient (case no. 2) with 4 ulcers was excluded at 4 months because of participation for other angiogenic trial and died at 7 months due to hyperkalemia on the day that the patient underwent peripheral artery bypass surgery. 2M, 6M, 12M, and 24M indicate 2, 6, 12, and 24 months, respectively, after the second injection.

Table 4. Serial Change in the Number of the Patients Classified by Fontaine Stage

	Fontaine Stage				Improve/Worsen (Details)	Discontinued (Details)
	II a	II b	III	IV		
Pre	0	7	4	11		
2M	0	9	3	10	Improved 2* (III to IIb 1, IV to IIb 1)	
6M	1	11	1	6	Improved 2† (IIb to IIa 1, IV to IIb 1)	Discontinued 3† (Consent withdrawal 1, operation in the treated limb 1, participation for other angiogenic clinical trial 1)
12M	1	10	0	5		Discontinued 3‡ (Death 1, rebypass operation in the treated limb 1, minor amputation 1)
24M	0	12	0	4	Improved 1§ (IV to IIb 1) Worsened 1§ (IIa to IIb 1)	

2M, 6M, 12M, 24M indicate 2, 6, 12, and 24, respectively, months after the second injection. *The number of the cases that improved between baseline and 2 months. †The number of the cases that improved or discontinued between 2 months and 6 months. ‡The number of the cases that discontinued between 6 months and 12 months. §The number of the cases that improved or worsened between 6 months and 12 months.

continuous improvement in clinical symptoms up to 2 years after gene therapy. In this study, 8 of 9 patients with CLI whose ABI was measurable achieved continuous improvements in rest pain and ulcers at 2 years. The ABI was maintained over 0.5 in 8 of 9 CLIs, although, in 7 of 9 ABI peaked out from 6-12 months to 24 months. It was suggested that in the patients whose ABI was over 0.5, local blood was sufficient to exceed the critical point to improve rest pain and ulcer. However, from this study, it is not clear how long the improvement in clinical symptoms would be continued over 2 years.

Table 5. Serious Adverse Events

Case No.	Serious Adverse Events	Days After 2nd Injection	Outcome	Causality
2	Death	226	Death	No
6	Cerebral infarction	26	Resolved	No
6	Hyperglycemia	173	Resolved	No
6	Infection	248	Resolved	No
8	Fever	130	Resolved	No
8	Fever	170	Resolved	No
8	Gastrointestinal bleeding	195	Resolved	No
8	Death	232	Death	No
9	Cataract operation	391	Resolved	No
17	Right toes minor amputation	267	Resolved	No
14	Infection of synthetic graft	137	Resolved	No
14	Left femur fracture	199	Resolved	No
16	Cataract operation	271	Resolved	No
20	Pneumonia	13	Resolved	No
20	Heart failure	46	Resolved	No
21	Pneumonia	13	Resolved	No
21	Heart failure	46	Resolved	No

Serious adverse events of case Nos. 20 and 21 were doubly counted because this patient was treated bilateraly at the same time.

It should be noted that 3 randomized placebo-controlled double-blinded studies (1 phase III trial in Japan and 2 phase II trial in the United States) successfully demonstrated the effectiveness of HGF gene therapy. HGF-STAT trial, a phase II randomized placebo-controlled double-blinded study, demonstrated that transcutaneous oxygen tension significantly increased at 6 months in the high-dose HGF group compared with the placebo, low-dose, and middle-dose groups.[19] Additional phase II study in the United States also demonstrated that change in toe brachial pressure index, as well as VAS, had significantly improved from baseline at 6 months in HGF-treated group compared with placebo.[20] More dramatic evidence of the clinical efficacy of HGF gene therapy was obtained from phase III study in Japan. In this study enrolling 44 patients, the overall improvement rate of the primary end point was 70.4% in the HGF group and 30.8% in the placebo group, showing a significant difference.[18] Especially, the HGF group achieved a significantly higher improvement rate (100%) than the placebo group (40%) to reduce ischemic ulcer.[18]

Up to 6 months after HGF gene therapy, all clinical studies demonstrated the safety of this treatment. In this study, even up to 2 years after HGF gene therapy, few safety concerns were documented. In this study, 1228 adverse effects based on good clinical practice guideline were reported during the 2 years. However, some of the moderate adverse effects, such as pain or small hemorrhages at the injected sites, were only considered

Table 6. Cumulative Rates of Major Amputation and Mortality

	Follow-Up Period, % (Events)			
Cumulative rate	0–6M	9M	12M	24M
Major amputation	0 (0)	0 (0)	0 (0)	0 (0)
Death	0 (0)	9.5 (2)	9.5 (2)	9.5 (2)
Number of analyzable patients	22	21	21	21

M indicates months.

to be related to gene therapy. But most of them were not considered to be related to gene therapy. Potential side effects, such as hemangioma, cancer, or worsening diabetic retinopathy, were not observed during the 2 years after transfection. Indeed, the previous study demonstrated no increase in the serum HGF concentration during gene therapy.[17] More exciting data of the present long-term follow-up study are the incidence of major amputation and mortality. Previous HGF-0205 trial (phase II) documented that complete ulcer healing at 12 months occurred in 31% of patients in the HGF group and 0% in the placebo group.[20]

In this study, major amputation of the treated limb with HGF plasmid was 0% through 2-year follow-up, or mortality was 9.5% at 12 months and up to 2 years (Table 6). In contrast, historical control studies demonstrated higher amputation and mortality rate. TransAtlantic Inter-Society Consensus-II stated that 1-year natural history of CLI included 30% major amputation and 25% death.[23] Data from control group of other angiogenic gene therapy could represent a natural history of nonrevascularized CLI. For example, phase II fibroblast growth factor gene therapy study showed 33.9% major amputation rate and 23.2% death rate at 1 year in the placebo group,[6] whereas in the phase III fibroblast growth factor gene therapy major amputation was 21% and mortality was 15% at 1 year.[7] However, it was reported that the age-adjusted major amputation rate was lower in Japanese population than in north of England and North America.[24] In Japan, although few reports mentioned the fate of CLI, Shigematsu et al[25] reported 15% major amputation rate and 18.5% mortality at 1 year, and Kumakura et al[26] reported 27.3% death rate at 1 year. Compared with these natural history data of CLI in Japan, in the current study, the rate of amputation-free survival and mortality in patients treated with HGF gene seemed to be favorable.

However, the long-term clinical outcome of angiogenic therapy using bone marrow mononuclear cells implantation reported that the mortality rate at 2 years was 20% and 0% in ASO and TAO group, respectively, whereas the amputation rate at 2 years was 33% and 9% in ASO and TAO, respectively.[27] Although the direct comparison is difficult because of the differences in the patient population, HGF gene therapy seems to be superior to cell therapy in event-free survival. In the cell therapy, ABI was not significantly improved, whereas ulcer size and rest pain (VAS) were reduced, and its effect was sustained up to 2 years. In the present study, HGF gene therapy significantly improved ABI up to 2 years. In the HGF study, baseline ABI (0.46) was lower than that in cell therapy (0.58 in ASO group). These results suggest that HGF gene therapy might be more suitable to treat patients with lower ABI.

This study has several limitations. First, this is not a randomized or placebo-controlled study. Second, this trial was the preliminary study before phase III randomized trial, and the number of involved patients was small. Thus, statistical values might be weak, even if P values were calculable and significant. Third, the long-term results from the present study might be biased because of some unanalyzable dropout patients. During the 2-year follow-up, there were 4 unanalyzable dropout cases: Nos 2, 4, 8, and 14, all of whom were patients with CLI, whereas the remaining group had 7 cases of intermittent claudication and 11 of CLI. The dropout group could have included more severe patients than the remaining group. Thus, it remains possible that more severe patients tend to drop out, and the results from the current study may have potential for bias to be overestimated.

Overall, the present study demonstrated that the 2-year long-term efficacy of phase I/IIa open-label study using intramuscular injection of naked HGF plasmid is safe, feasible, and might achieve successful improvement in ischemic limbs as sole therapy. Larger studies to determine whether HGF plasmid can avoid major amputation and decrease the mortality in patients with CLI are warranted.

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Disclosures

Ryuichi Morishita is a member of the Board of AnGes MG that has developed the HGF gene therapy drug and has stocks of AnGes MG. Part of the information included in this manuscript has been previously published in *Hypertension,* 2004, and *Arteriosclerosis Thrombosis Vascular Biology*, 2011.

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Overexpression of endothelin-1 and endothelin receptors in the pulmonary arteries of failed Fontan patients

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ABSTRACT

Background: Endothelin-1 (ET-1), a potent vasoconstrictor, is considered to be implicated in failing Fontan circulation, however the expressions of ET-1 and endothelin receptor type A (ET_AR) and type B (ET_BR) in the pulmonary arteries of failed Fontan patients were not elucidated.
Methods: Immunohistochemistry and quantitative real-time PCR were used to analyse the expression levels of ET-1 and its receptors in the pulmonary arteries of the autopsy lung tissues of the patients who died after the Fontan procedure (n=10). We divided these patients into 3 groups, failed Fontan (n=4), heart failure (n=3) and non-failed Fontan (n=3), and then compared those to the age-matched normal controls (n=4).
Results: The intra-acinar pulmonary arteries of failed Fontan patients showed significant medial hypertrophy. Computational optical density analyses of the immunostaining revealed that the expressions of ET-1, ET_AR, and ET_BR in the intra-acinar pulmonary arteries were significantly increased in the failed Fontan patients ($P<0.05$ vs. normal controls), however no significant difference was observed between the non-failed Fontan patients and the normal controls. Quantitative real-time PCR analyses confirmed that the mRNA expressions of ET-1, ET_AR, and ET_BR were significantly increased in the failed Fontan patients ($P<0.05$ vs. normal controls).
Conclusion: The overexpression of ET-1 and its receptors in the pulmonary arteries can cause pulmonary vasoconstriction and vascular remodelling, leading to failed Fontan circulation. This study suggests a histopathological rationale for the potential benefits of endothelin receptor antagonists in patients with failing Fontan circulation.

1. Introduction

The Fontan procedure has been a landmark contribution to the therapeutic strategies for congenital heart disease with single ventricle physiology. Although the mortality after the Fontan procedure has decreased, some patients still show failure of the Fontan circulation, such as protein-losing enteropathy, plastic bronchitis, severe cyanosis, and low cardiac output, during short-, mid-, and long-term follow-up. This can result in takedown, cardiac transplantation, and death [1–4]. The outcome of the Fontan procedure depends on several factors, including pulmonary vascular resistance, ventricular function, atrioventricular valve regurgitation, thrombosis, and arrhythmias. Among these, pulmonary vascular resistance is the predominant factor for a good Fontan circulation because even a slight elevation in pulmonary vascular resistance leads to subsequent low cardiac output [5,6]. Previous histomorphological studies revealed that significant medial hypertrophy of the pulmonary arteries was correlated with poor outcome of the Fontan procedure [7,8]. Several vasoconstrictive substances derived from the endothelium have been shown to induce medial hypertrophy and increase pulmonary vascular resistance by promoting the proliferation of vascular smooth muscle cells. Endothelin-1 (ET-1) is one of the most potent vasoconstrictive substances, which can increase pulmonary vascular resistance and also has mitogenic properties. Previous studies demonstrated that circulating ET-1 levels were elevated in patients with various types of pulmonary arterial hypertension and even in patients who underwent a Fontan procedure, and that circulating ET-1 levels were correlated with the parameters of the pulmonary haemodynamics in the Fontan circulation [9,10]. ET-1 acts through 2

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